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13. ABSTRACT (Maximum 200 words) <i>Myxococcus xanthus</i> is a soil bacterium that forms spore-filled fruiting bodies in response to nutrient limitation coupled with high cell density. Extracellular A-signal, which is composed of amino acids and peptides, is produced during early development and is thought to function as a cell density signal. The <i>asgC</i> gene is one of three known regulatory genes that are required for A-signal production. The <i>asgA</i> and <i>asgB</i> genes encode a signaling kinase and a putative transcription factor, respectively. The goal of this project is to understand the role of <i>asgC</i> in A-signal production. To this end, the DNA sequence of <i>asgC</i> and the only known <i>asgC</i> mutant allele, <i>asgC767</i>, was determined. The <i>asgC</i> gene encodes RpoD, the major sigma factor in <i>M. xanthus</i>. The second aim is to isolate pseudorevertants of <i>asgC767</i> that have regained the ability to produce fruiting bodies and extracellular A-signal. Approximately 40 independent pseudorevertants have been isolated, and their characterization has been initiated. Analysis of these mutants may lead to the identification of additional components of the signal transduction pathway leading to A-signal production. In addition, we are exploring the role of <i>AsgC</i> in expression of <i>celA</i>, a gene encoding an extracellular endoglucanase.				
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(4) STATEMENT OF THE PROBLEM STUDIED:

Myxobacteria are unique prokaryotes in that they display a range of multicellular behaviors requiring cell-cell interactions (for review, see (Shimkets, 1990)). Perhaps the most impressive social behavior of the myxobacterium *Myxococcus xanthus* is the building of multicellular, haystack-shaped structures known as fruiting bodies. Upon starvation, many thousands of cells glide to aggregation centers where they participate in the formation of a fruiting body. Cells within the nascent fruiting body differentiate into ovoid, heat- and desiccation-resistant myxospores. The long-term goal of this research is to determine how individual cells sense starvation and regulate production of an extracellular signal (A-signal) required during the initiation of multicellular development.

The *asg* mutants

The *asg* (A-signal generating) mutants of *M. xanthus* fail to generate extracellular A-signal and are arrested early in development. As a postdoctoral fellow in Dr. Dale Kaiser's laboratory, I carried out biochemical studies to purify and characterize A-signal. My colleagues and I showed that A-signal is composed of amino acids and peptides, and it is generated by extracellular proteases (Kuspa et al., 1992, Plamann et al., 1992) . A high cell density is required for fruiting body formation, and we propose that sensing of extracellular amino acids provides a way for *Myxococcus* to determine the cell density of the starving population of cells (Kuspa et al., 1992) .

There are three known *asg* loci: *asgA*, *asgB*, and *asgC* (Kuspa and Kaiser, 1989) . The overall goal of the work in my current laboratory is to understand the roles of the *asg* genes in A-signal production and starvation sensing. The *asgA* and *asgB* genes have been studied as part of another project. A summary of these studies is given below, and provides the context for evaluation of the work described in this final progress report. **The purpose of this project was to characterize *asgC* as well as uncover additional components of the signal transduction pathway(s) required for sensing of starvation and A-signal production in *M. xanthus*.**

asgA

The deduced amino acid sequence of AsgA was found to have a remarkable similarity to members of the histidine protein kinase family and the response regulator family of the so-called "two-component" regulatory systems. In the paradigm two-component system, the histidine protein kinase domain or "transmitter" is part of a transmembrane sensor protein, and the response regulator domain or "receiver" is part of a transcriptional regulator protein. When the sensor is stimulated by ligand binding, it is autophosphorylated by its histidine protein kinase domain. The phosphoryl group is transferred to the receiver domain of the transcriptional regulator; phosphorylation modulates the activity of the DNA binding domain and ultimately results in a change in gene expression (Parkinson and Kofoed, 1992) .

In most of the two-component signal transduction systems, the transmitter and receiver domains are located on separate polypeptides (Parkinson and Kofoed, 1992) . AsgA is somewhat unusual because it consists entirely of a receiver domain at the N-terminus followed by a transmitter histidine kinase domain at the C-terminus. AsgA appears to lack the hydrophobic, membrane-spanning regions that characterize most of the sensors, and, therefore, is likely to function in the cytoplasm. Because AsgA appears

to lack "input" and "output" domains (that is, domains that interact with a signaling ligand or bind to DNA), it is likely to interact with other signaling proteins that have input or output functions. AsgA may function in the middle of a phosphorelay similar to the phosphorelay that controls initiation of sporulation in *Bacillus subtilis* (Burbulys et al., 1991, Grossman, 1991)

asgB

The deduced 163 amino acid sequence of AsgB contains a region similar to conserved region four of sigma factors (Plamann et al., 1994) . Region four is characterized by its location at the C-terminus and a conserved helix-turn-helix structure that directly contacts the -35 region of promoter sequences (Dombroski et al., 1992, Gardella et al., 1989, Lonetto et al., 1992, Siegele et al., 1989) . There is no evidence for the presence within AsgB of region two, a second highly conserved region that is believed to interact with core polymerase and the -10 regions of promoters; therefore, it is unlikely that AsgB is a sigma factor. Instead, AsgB may function as a transcriptional activator or repressor.

DNA sequence analysis of *asgC* (The remaining work described in section 4 was funded by the ARO from 1996-1998)

We cloned *asgC* and localized the gene to a region of the *M. xanthus* chromosome that contains genes homologous to those of the *E. coli* macromolecular synthesis (MMS) operon. The MMS operon contains genes that are necessary for the initiation of translation (*rpsU*), DNA replication (*dnaG*), and RNA synthesis (*rpoD*). The first gene of the operon, *rpsU*, encodes the ribosomal protein S21, which interacts with the 16S ribosomal RNA sequences complementary to the ribosome-binding sites of mRNAs. Initiation of DNA replication requires primase, the product of *dnaG*, the second gene of the MMS operon. Primase is responsible for synthesis of RNA primers that are required for the production of lagging-DNA strand Okazaki fragments (Lupski and Godson, 1984, Versalovic et al., 1993) . The *rpoD* gene encodes the major sigma factor, which is responsible for promoter sequence recognition during initiation of mRNA synthesis. A nested set of deletion plasmids derived from a clone containing the region homologous to the MMS operon was constructed using exonuclease III. By analyzing the ability of these plasmids to rescue development of the *asgC* mutant, we localized the *asgC767* mutation to within the *rpoD* gene. We cloned *asgC767*, the only known *asgC* mutant allele, and found that it contains two adjacent transition mutations (G to A), resulting in a glutamate to lysine substitution at position 598 of RpoD. This substitution lies within region 3.1, which contains many acidic residues and is conserved among sigma-70-like sigma factors (Lonetto et al., 1992) .

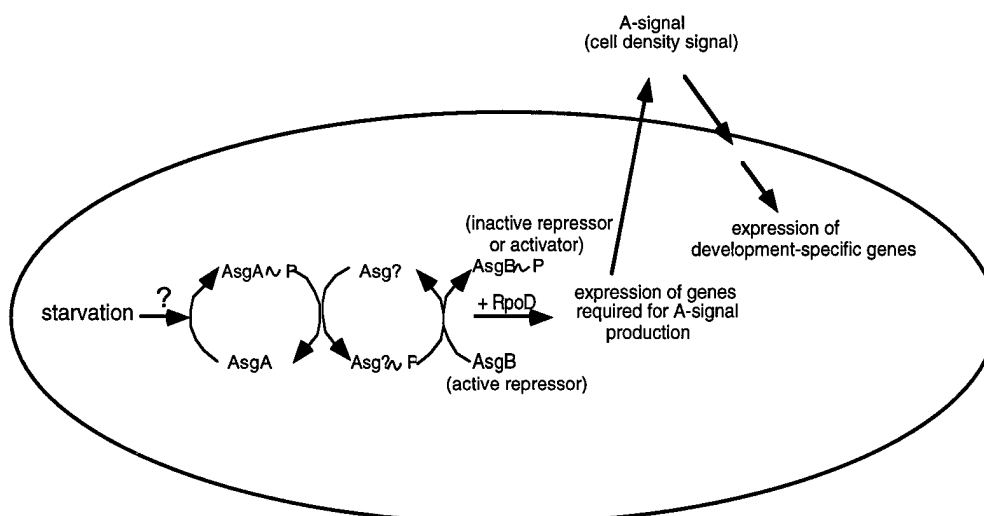
The *asgC767* (*rpoDEK598*) mutant does not appear to have a general defect in growth, and its vegetative phenotypes (tan rather than yellow colony color, decreased cohesiveness, and decreased extracellular enzyme production) are very similar to those of the *asgA* and *asgB* mutants. We found that, as in *asgA* and *asgB* mutants, expression of $\Omega 4521$ is reduced during growth and development in the *asgC767* background. Furthermore, expression of $\Omega 4521$ in the *asgC* mutant is restored upon addition of A-signal. These observations are consistent with the hypothesis that the *rpoD*(*EK598*) mutant has a defect in A-signaling that is similar to the defect of the *asgA* and *asgB* mutants, rather than a more general defect in transcription initiation.

Model for the role of AsgC (RpoD) during development

One hypothetical explanation for the A-signaling defect observed in the *rpoD(EK598)* strain is that the mutant sigma subunit fails to productively interact with a transcriptional regulator that affects A-signal production. Mutations that affect interactions between RNA polymerase and transcription factors have been identified in the genes encoding the alpha and sigma subunits of RNA polymerase (Ishihama, 1993). *E. coli rpoD* mutations that affect interactions with the transcription factors PhoB (Makino et al., 1993), CRP (at the *P_{l gal}* promoter) (Kolb et al., 1993), AraC (Hu and Gross, 1985), and the λ cI repressor (Kuldell and Hochschild, 1994, Li et al., 1994) have been localized to region 4. Kumar et al. (Kumar et al., 1994) suggest, from data provided through a deletional analysis of the C-terminal portions of *rpoD*, that a region extending from at least region 3.2 to upstream of region 4.2 may be involved in association with transcription factors. More recently, Bramucci et al. (Bramucci et al., 1995) identified a mutation in a *B. subtilis* sigma factor gene (*spo0H*) that suppresses the transcriptional defects of a mutant form of the transcription factor Spo0A (Spo0A9V). This *spo0H* mutation is located between the sequences encoding regions 2 and 3 and is proposed to allow Spo0A9V to interact with the mutant sigma-H, restoring transcriptional activation. Similarly, the A-signaling defect observed in the *rpoD(EK598)* strain may be caused by a failure of the mutant sigma subunit to functionally interact with a transcriptional regulator necessary for A-signal production. If this hypothesis is correct, the isolation and characterization of suppressors of *rpoD(EK598)* may allow identification of the hypothetical transcriptional regulator or its target genes. An alternative hypothesis is that the mutant sigma factor has a greater affinity for core polymerase, preventing alternative sigma factors necessary for A-signal production from gaining access to core. In this case, a suppressor analysis may result in the identification of genes encoding alternative sigma factors or RNA polymerase mutants that have altered affinities for sigma subunit.

A model for the A-signal-generating pathway

The figure below illustrates one of many models for the role of the *asg* gene products in A-signal generation. Given that the *asg* mutants have very similar phenotypes and the *asg* genes encode proteins that appear to have regulatory functions, we hypothesize that the *asg* gene products function together in a regulatory pathway that is required for production of extracellular A-signal. An alternative model is one in which the *asg* gene products function in different, but related, regulatory pathways. In the figure, AsgA (a histidine protein kinase) is shown interacting with an unknown starvation sensor, which may be another histidine protein kinase, a serine/threonine kinase, or a small-molecule phosphodonor. This interaction results in activation of the AsgA autokinase, and AsgA is converted to AsgA-phosphate. The phosphoryl group is then transferred to an unidentified protein or through a series of proteins, and finally to AsgB (a putative transcription factor). In this model, AsgB acts as a repressor of early developmental genes when it is nonphosphorylated and as an activator of these genes when phosphorylated. Expression of the genes required for A-signal production depends upon wild type sigma factor, perhaps for interaction with phosphorylated AsgB and/or ppGpp. Expression of these genes, which may include protease genes, results in extracellular A-signal production. Finally, sensing of A-signal leads to the expression of development-specific genes such as $\Omega 4521$. It is easy to imagine several variations of this model. For example, the regulator at the end of the pathway may be a transcriptional activator whose production is regulated by AsgB, or AsgA may function downstream rather than upstream of AsgB.



A model for the A-signal-generating pathway (see text).

Isolation and characterization of pseudorevertants of the *asgC767* mutant

The isolation and characterization of pseudorevertants is a powerful genetic approach that was used to provide insight into the A-signal generating pathway. Secondary mutations that reverse the mutant phenotype, in this case, the inability to form fruiting bodies and sporulate, are known as "suppressor" mutations (Botstein and Maurer, 1982). With the exceptions of informational suppression and back mutation, the manner by which the mutant phenotype is reversed can tell us much about the function of the mutant gene. Bypass suppressors, which are gene-specific but not allele-specific, act on null mutations as well as missense mutations because the original mutant protein is not required for suppression (Parkinson, 1995). For example, bypass of the *asgC* defect may occur if the cell acquires a mutation that obviates the need for A-signal to turn on A-signal dependent genes during development. In short, some bypass suppressors will identify downstream components of the A-signal-generating pathway, whereas others are likely to provide information about how the pathway operates without identifying pathway components.

Conformational suppressors reverse the mutant phenotype by restoring activity of the mutant protein. Activity may be restored following a change at a secondary site within the mutant protein that alters its folding, stability, or function. Of greater interest with respect to this study, activity may be restored by a compensating change in a protein that interacts with the original mutant protein. Isolation of this type of interaction suppressor, which is highly allele-specific, requires the presence of stable mutant protein. Interaction suppressors of *asgC* would identify components of the A-signal generating pathway that lie immediately upstream or downstream of *AsgC*.

We have isolated *asgC* pseudorevertants that form fruiting bodies and sporulate. A protocol described by Rhie and Shimkets (Rhie and Shimkets, 1989) was used. In brief, *asgC* mutant cells were UV-mutagenized, and plated on clone-fruiting (CF) medium, a medium that supports development of wild type cells but not *asg* mutants. The plates were incubated for 3 to 5 days at 32°C, and then were heat-treated to kill vegetative cells. Heat-treated cells were resuspended in buffer, subjected to sonication (to disrupt any remaining vegetative cells and break up clumps of spores), and plated on a

rich medium. Colonies were then tested for fruiting body formation on starvation plates and/or CF medium. Only one pseudorevertant/mutagenesis was saved for further analyses, unless pseudorevertants with obviously different phenotypes were observed.

We have isolated approximately 100 *asgC* pseudorevertants from 39 independent selections. We have found that about 2/3 of our *asgC* suppressors are linked to *asgC*. These suppressor mutations are most likely intragenic suppressor mutations or reversions. For these studies, we have concentrated on those pseudorevertants that contain suppressor mutations that are unlinked to *asgC*.

Analysis of A-signal production by the pseudorevertants

We have used the standard A-signal bioassay to determine whether the pseudorevertants have restored A-signal production. So far, we have determined that there are at least three classes of pseudorevertants with respect to A-signal production: One class produces wild type levels of A-signal, a second class produces no A-signal (a level comparable to that of the original *asgC767* mutant), and a third class produces an intermediate level of A-signal. The mutants that produce little or no A-signal appear to have bypassed the developmental requirement for A-signal.

(5) SUMMARY OF THE MOST IMPORTANT RESULTS

During this final reporting period, we initiated experiments to explore the recent finding that an endoglucanase gene (*celA*) is regulated by *asgC* (Bensmail *et al.* 1998). We chose to explore this finding because it relates to the long-term goal of this project, which is to understand the role of *asgC* (*sigA*) in the A-signal production pathway. The *asg* mutations, including the only known mutation in *asgC*, cause a decrease in the level of extracellular enzyme production during growth, as well as a decrease in the enzymes that generate extracellular A-signal during early development. Although the *asg* genes have been proposed to function in a signal transduction pathway that regulates expression of genes encoding extracellular enzymes, specific gene targets for the regulatory pathway have not been identified.

Bensmail *et al.* (1998) found that in *asgA* and *asgC* mutants, the level of *celA* message is reduced during growth (The group was unable to extract RNA from the *asgB* mutant strain, so the level of *celA* mRNA remains unknown in this strain). We obtained a plasmid containing the *celA* gene, and have constructed a *celA-lacZ* fusion. We did this by first obtaining PCR primers that hybridize to sequences that flank an approximately 350 bp region upstream of the *celA* orf, and that each contain a *Bam*HI recognition sequence at its 5' end. The primers were used to amplify the *celA* upstream region; the PCR product was digested with *Bam*HI and then inserted into the *Bam*HI site of plasmid pKS.kla (kindly provided by Heidi Kaplan), which resulted in the formation of a transcriptional fusion of *celA* to *lacZ*. We sequenced across the insert junctions to verify that the insert is in the correct orientation. We transformed *M. xanthus* strains (wild type, as well as *asgC*, *asgB*, and *asgA* mutants) with the *celA-lacZ* fusion plasmid. Cultures of transformed cells were placed under growth and developmental conditions, and the β -galactosidase activities within the cells was measured. If the *asgC* gene regulates *celA* as reported by Bensmail *et al.*, we would expect that the level of β -galactosidase activity to be low (relative to the level in wild type cells) in the *asgC* mutant cells. However, our results provided no evidence that *celA* is regulated by the *Asg* genes. After further examination of the data presented by Bensmail *et al.*, in particular the primer extension data, we think it is likely that the promoter for *celA* is located further upstream than was reported. We are now planning to amplify *celA* to include more upstream sequences in an attempt to locate the putative *Asg* regulatory sequences

Identification of a gene that is regulated by *asgC* (and is negatively affected by the *asgC767* mutation) will provide us with a valuable tool for future genetic and molecular biological studies of the roles of the *asg* genes in extracellular A-signal production. For example, the *celA* upstream DNA could be used in *in vitro* studies to look for direct interaction between the Asg proteins and the *celA* promoter. In addition, expression of the *celA-lacZ* fusion gene could be monitored in the *asgC* suppressor strains to identify more precisely the point at which suppression occurs.

(6) LIST OF ALL PUBLICATIONS

The initial proposal for this research was submitted to the ARO in August, 1994. However, funding was delayed until 15 April, 1996. The following manuscript resulted from the work that was carried out during the intervening period (see Appendix A).

Papers published in peer-reviewed journals:

Davis, John M., Jocelyne Mayor, and Lynda Plamann. 1995. A missense mutation in *rpoD* results in an A-signaling defect in *Myxococcus xanthus*. *Molecular Microbiology* **18**:943-952.

Dunmire, Valerie, Laura Tatar, and Lynda Plamann, "Genetic Suppression Analysis of an *asgA* Missense Mutation in *Myxococcus xanthus*," *Microbiology*, 145:1299-1306, 1999.

Book chapters:

Plamann, Lynda and Heidi B. Kaplan. 1998. Cell-density sensing during early development in *Myxococcus xanthus*. In G. Dunney and S. Winans (ed.), *Cell-cell communication in bacteria*. American Society for Microbiology, Washington, DC.

(7) LIST OF ALL PARTICIPATING SCIENTIFIC PERSONNEL

Lynda Plamann, Ph.D. (P.I.)

1996-1997:

John M. Davis (graduate student)

Rina Nop (part-time research assistant)

Laura Arthur (undergraduate researcher)

1998-1999:

Amy Cunningham (research assistant)

(8) REPORT OF INVENTIONS

none

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(10) APPENDIXES